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The Bacteriophage Reaction For the Identification of Bacteria

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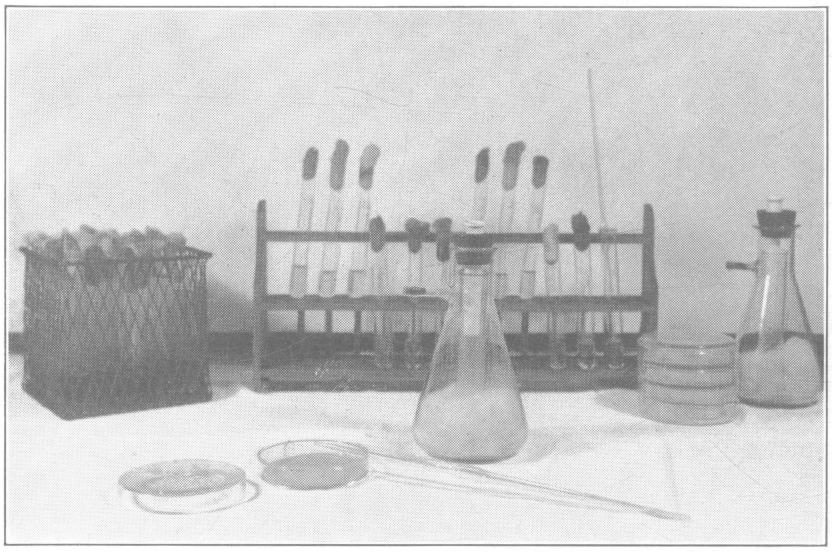

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CONTENTS

THE BACTERIOPHAGE REACTION FOR THE IDENTIFICATION OF BACTERIA

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Various biochemical and physiological procedures such as reactions to dyes, carbohydrates, and specific sera have been useful for the identification of bacteria. The morphology of micro-organisms cannot be disregarded, yet it furnishes only general information. The use of a specific serum developed in the blood of an animal has afforded the most satisfactory results. The identity of a species of bacteria can be demonstrated readily by the agglutinating effect of the serum upon a suspension of the bacteria.

Because few plant pathological laboratories are equipped to use the serum method and because it is not possible at times to secure a specific serum for a given organism even in laboratories that are equipped to develop it, the use of seriological techniques has always been limited. A simpler method, such as the phage reaction, will find much wider favor.

Plant pathologists have usually employed plant inoculations to establish identity of a bacterial organism and cultural reactions to aid in the description. These operations involve considerable time. In the identification of bacteria there is a great need for a quick, reliable method that requires little equipment and is within the compass of all laboratories.

The basis of the bacteriophage reaction is the lysis of bacteria or the inhibition of their growth. Phage reactions are as striking and as definite as are agglutination and precipitin reactions. Bacteriophages are remarkably specific.

In this paper, the technique for developing phages from water extracts of green plants and seeds is outlined, the specificity of phages is demonstrated, and some precautions which must be observed in interpreting results are pointed out.

Origin of Bacteriophages

Nearly all early reports regarding bacteriophages, beginning with Twort and d'Herelle (4, 9), have indicated that the most common sources of their phages were river water which contained sewage, fecal material from convalescent patients, pus from wounds, or decaying vegetables and plant tissue (2). All of these sources involve very complex mixtures.

The writer discovered and reported (7, 8) several years ago that water extracts of green plants, seeds, and grains are excellent sources of phages for many species of bacteria. Of course phages cannot be elaborated for all forms of bacteria from any one extract. Oat grain extract has been used most frequently. Phages for about 30 different species of bacteria, comprising both plant and human pathogens as well as saprophytes, have been prepared with the use of oat extract. Although all the phages were initiated from a common source, each reacted best, and usually only, with its own homologous organism.

The active principle in extracts which gives rise to phages or lytic factors has been designated as a phage-precursor. The writer uses the term precursor in a different sense from that employed by Krueger (6). This precursor, as it occurs in extracts, is nonspecific, yet when introduced into a culture of susceptible bacteria, a phage specific for the culture is developed. No explanation can be advanced at the present time to account for this phenomenon. These lytic factors developed from seed and plant extracts appear to be true phages. They are transmissible in series indefinitely in liquid media and, in the presence of susceptible cultures, are demonstrated by the appearance of "plaques" upon solid media. Phages elaborated for a number of different species of bacteria from a water extract of oat grain vary in size of plaques, time of action, and degree of lysis even as those which can be obtained from other sources. Yet, there is one difference. *A phage developed for a particular species of bacteria from a certain grain or plant extract is always the same.* This fact is believed to be of considerable importance in the use of phages for identification purposes.

Another valuable source of phage for a pathogenic bacterium is a water extract of a dead plant which has succumbed as a result of infection. Usually, a phage will be found sooner or later after infection in the most susceptible plants. The writer has been successful in obtaining phages for *Xanthomonas phaseoli* and *Corynebacterium sepedonicum* only from dead diseased plants. No seed extracts or tissues of living plants have been found which contained the necessary phage-precursors for these organisms. A water extract of dead, diseased plant tissue contains a phage specific for the organism which caused the death of the plant. This distinction is made because an extract of healthy plant tissue, or of seeds or grains, contains a nonspecific phage-precursor which does not become a phage specific for a species of bacteria until added to a culture of the organism. In this connection, it is particularly interesting to consider in the tables which are shown later that specific

phages can be developed from a water extract of oat grain for such widely varying species of bacteria as *Xanthomonas pruni, Erwinia amylovora, Vibrio cholera, Escherichia coli,* and *Corynebacterium fasciens.*

Technique for Developing Phages

Since oat grain extract has been used most frequently and has been found to be useful for developing phages for many organisms, the technique developed for that extract will be outlined.

Wash 100 grams of oats several times with water to remove dirt and extraneous matter. This can be done by adding 300 milliliters of distilled water to the grain in a flask or beaker. After stirring or shaking for a few minutes, drain off the liquid by pouring on a wire screen of sufficiently small mesh to retain the oats. After washing, add 125 to 150 milliliters of distilled water and set aside to soak. Soaking at room temperature of 18° C. for 24 hours or at 8° C. in a refrigerator for 2 or 3 days is sufficiently long to produce a satisfactory extract. After extraction, the brown-colored liquid is removed by filtering.

The quality of the extract for phage production is improved by placing in the freezing compartment of a refrigerator and reducing the liquid about one-half in volume. The extract may be rendered more active also by dialyzing against distilled water until the color has been removed. The reason for extraction at a relatively low temperature is to control microbial activity else the phage-precursor may be lost. After either treatment, a phage may be demonstrated for a susceptible organism in the first or second passage, whereas with the original untreated extract five or six serial passages may be required.

After the oat extract has been prepared it is then filtered through a sterile porcelain candle sufficiently fine to remove bacteria. A liquid medium is always used in developing a phage. Any medium is satisfactory in which the organism for which a phage is desired grows well. Usually, a series of three tubes, each containing 8 milliliters of nutrient solution, is used. All are inoculated with three drops from an 18- to 24-hour-old culture. The first tube is designated as a check. To the second and third tubes the filtered oat extract is added in quantities of three drops to the second and one milliliter to the third tube. The cultures are then incubated at a temperature optimum for growth. After 18 to 20 hours, the check tube will show normal growth while the tubes to which the extract was added will be completely clear or only slightly clouded (fig. 1). If clouded, the liquid in tubes 2 and 3 should be filtered to remove bacteria and a new series started, using the filtrate from

the first series but reducing the amount of filtrate added to the second and third tubes to 3 and 7 drops. Volumetric pipettes of 1- or 2-milliliter capacity delivering 20 to 25 drops per milliliter are used.

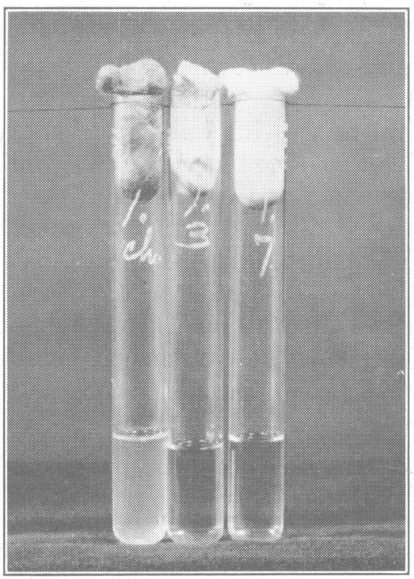

Fig. 1.—The check tube (left) shows normal growth, while the phage treated tubes are clear—a 4-plus lysis.

In the development of a phage specific for a culture at least six or more series of cultures should be made. This must be done to remove by dilution the uncombined, nonspecific phage-precursor of the original extract, else a false reaction may be obtained when the filtrate is used for the identification of an unknown isolate. If a 4-plus lysis is attained in the first or second passage, it is not necessary to filter before starting another series.

Use of the Phage for Identification

Only phages which effect complete lysis of a culture are satisfactory to use for identification. Complete clearing of a culture is conveniently designated as 4-plus lysis. Phages which yield only partial lysis are likely to cause errors in the estimation of the amount of clearing produced, unless a turbidimeter is used. This, of course, is not necessary with phages showing complete lysis.

If one plant or grain extract does not contain a phage-presursor which will result in the development of a phage showing complete lysis of an organism, extracts of other grains or seeds should be tried. With our present incomplete knowledge of phage origin and action there is no way to predict where a phage-precursor for a given organism may be found. However, seeds of grasses and cereals, so far, have been our best source.

Nutrient broth is a satisfactory medium for nearly all plant pathogens. After a phage has been developed for a known organism, new isolates of the same organism may be identified by testing against a phage for that species. Although a known culture of the

organism is desirable for comparison, it is not necessary if one is familiar with the action of the phage used. This involves a definite knowledge of the degree, time, and duration of lysis produced. Some phages appear to stimulate bacterial growth before lysis occurs, others do not. In some cases, lysis is complete within 4 hours, while in others, 18 to 24 hours are required.

In employing the phage reaction for the identification of unknown isolates, the author uses a set of three tubes for each culture to be determined. All tubes are inoculated alike from an 18- to 24-hour-old culture. One tube is reserved for a check; to the second and third tubes, one and three drops of phage filtrate are added. The cultures are then placed in an incubator at optimum temperature for growth. Usually, after 5 to 10 hours, a positive phage reaction becomes evident, as shown by the normal clouding of the check tube and the complete absence of growth in the tubes containing phage filtrate. Isolates which show the same degree of lysis as the known culture used for comparison may be considered to be identical with it. The lytic reaction is of value only with pure cultures; that is, those which develop from a single colony or a single organism.

A modification of the phage method of identification may be used to advantage in some instances. In addition to testing a phage prepared for a known species of bacteria against a new isolate, develop a phage for the isolate and determine if the known species will react with it.

Reaction of Bacteria to Specific and Nonspecific Phages

When a group of organisms is tested against phages developed for other organisms, it is found that each phage reacts best with its own homologous species or strain. Typical demonstrations are shown in tables 1, 2, and 3.

TABLE 1.—Homologous bacteriophages*

Species of Bacteria	1	2	3	4	5	6	7	8	9	10
1. *Xanthomonas pruni*	4+	—	—	—	—	—	—	—	—	—
2. AC-X	—	4+	2+	—	—	4+	—	—	—	—
3. *Erwinia amylovora*	—	+	4+	—	—	—	—	—	—	—
4. *Pseudomonas glycinea*	—	—	—	4+	—	—	—	—	—	—
5. *Corynebacterium sepedonicum*	—	—	—	—	4+	—	—	—	—	—
6. *Corynebacterium michiganense*	—	3+	—	—	—	4+	—	—	—	—
7. *Agrobacterium tumefaciens*	—	—	—	—	—	—	4+	—	—	—
8. *Pseudomonas syringae*	—	—	—	—	—	2+	—	4+	—	—
9. *Xanthomonas campestris*	—	—	—	—	—	—	—	—	4+	—
10. *Corynebacterium fasciens*	—	—	—	—	—	—	—	—	—	4+

*4+ signifies complete lysis of a culture, while +, 2+, and 3+ indicate varying degrees of partial lysis. No reaction is represented by — .

TABLE 2.—Bacteriophages for plant pathogens*

Species of Bacteria	*Xanthomonas pruni*	AC-X	*Erwinia amylovora*	*Pseudomonas glycinea*	*Corynebacterium sepedonicum*	*Corynebacterium michiganense*	*Agrobacterium tumefaciens*	*Pseudomonas syringae*
Staphylococcus aureus	—	—	—	—	—	—	—	—
Aerobacter aerogenes	—	2+	—	—	—	—	—	—
Vibrio cholera	—	—	—	—	—	—	—	—
Shigella paradysenteria	—	—	—	—	—	—	—	—
Salmonella aertrycke	—	—	—	—	—	—	—	—
Salmonella schottmulleri	—	—	—	—	—	—	—	—
Salmonella paratyphi	—	—	—	—	—	—	—	—
Escherichia coli	—	—	—	—	—	—	—	—

*4+ signifies complete lysis of a culture, while +, 2+, and 3+ indicate varying degrees of partial lysis. No reaction is represented by — .

TABLE 3.—Bacteriophages for human pathogens*

Species of Bacteria	*Staphylococcus aureus*	*Aerobacter aerogenes*	*Vibrio cholera*	*Shigella paradysenteria*	*Salmonella aertrycke*	*Salmonella schottmulleri*	*Salmonella paratyphi*	*Escherichia coli*
Xanthomonas pruni	—	—	—	—	—	—	—	—
AC-X	—	—	3+	—	—	3+	2+	—
Erwinia amylovora	—	3+	—	—	—	—	—	—
Pseudomonas glycinea	—	—	—	—	—	—	—	—
Corynebacterium sepedonicum	—	—	—	—	—	—	—	—
Corynebacterium michiganense	—	—	2+	—	2+	2+	3+	—
Agrobacterium tumefaciens	—	—	—	—	—	—	—	—
Pseudomonas syringae	—	—	—	—	—	—	—	—
Xanthomonas phaseoli var. *sojense*	—	—	—	—	—	—	—	—
Xanthomonas phaseoli	—	—	—	—	—	—	—	—
Corynebacterium flaccumfaciens	—	—	—	—	—	—	—	—
Erwinia atroseptica	—	—	—	—	—	—	—	—

*4+ signifies complete lysis of a culture, while +, 2+, and 3+ indicate varying degrees of partial lysis. No reaction is represented by — .

In table 1, nine plant pathogens, tested against each respective phage, show that each phage reacts best with the species for which it was developed. In this series one exception is to be noted. The culture designated as AC-X was found in association with *Erwinia amylovora* in an apple canker. This organism reacted, not only with its own phage, but also with the one for *Corynebacterium michiganense*, both giving a 4-plus lysis. Reciprocally, there was a difference. The phage for AC-X showed only a 3-plus lysis when tested against *Corynebacterium michiganense*. This fact, together with differences in color and type of growth of the two cultures, should prevent error in using a phage for determining the identity of either organism.

When a group of eight human pathogens was tested against a similar number of phages for plant pathogens, reactions were all negative (table 2). Likewise, (table 3) when the phages for human pathogens were tried with species of bacteria isolated from plants, there were no instances of 4-plus lysis. No explanation can be offered at the present time regarding the significance of the 2-plus and 3-plus reactions. Possibly some kinds of relationships are indicated. Such weak reactions should not cause any confusion or invalidate the use of a phage for identification purposes.

Preservation of Filtrates

Bacteriophages may be prepared and stored for several years. Two methods have been followed in this laboratory. Brown glass bottles of 30-milliliter capacity, with bakelite screw caps, (fig. 2)

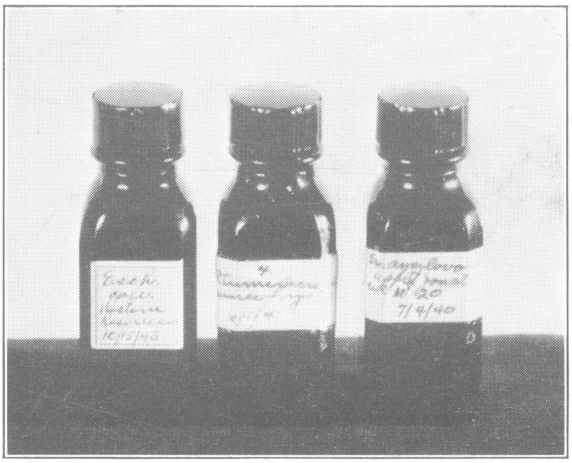

Fig. 2.—Bottles of this type are convenient for storing bacteriophage filtrates.

have been found to be satisfactory. These can be sterilized in a hot-air oven then filled with filtrate from a phage-treated culture and stored in a refrigerator at 8° C. Such filtrates have been kept for 6 years and still found to be fully active.

Hermetically sealed glass ampules containing from 1 to 10 milliliters are also useful. If the filtrate is warmed to 40° or 45° C. before placing in the ampule (which is immediately sealed with a blow torch), a partial oxygen tension is formed upon cooling. The ampules may be stored in the dark at room temperature. A phage filtrate for one strain of *Bacterium stewarti* was placed in a sealed tube 12 years ago. The phage is still active against the strain of *Bacterium stewarti* for which it was prepared and showed no loss of lytic activity.

Discussion

The use of bacteriophages for identification purposes is not a new idea. Evans (5) made use of them in studies on certain hemolytic streptococci and states that one would not go far wrong in depending upon phages for determining the identity of different species of the group included in her studies. Craigie and Yen (3) have reported a method for typing new isolates of *Eberthella typhosa*. The basis of the method is the specificity of a phage for the Vi form of the typhoid bacillus. By the use of this phage, new isolates of *Eberthella typhosa* may be classified quickly, thus determining not only the species but also the strain of the species.

The fact that phages can be developed for many different species of bacteria from grain and plant extracts has not been appreciated fully and may explain why the use of phages by plant pathologists for purposes of identification has not gained general practice.

Primary species of bacteria, that is, those which do not have phages or transmissible lytic factors associated with them, lend themselves readily to phage development. Secondary cultures, those which already contain a lytic factor, act differently. A phage cannot be prepared for them so easily. If there is difficulty in developing a phage for a culture, two reasons for the failure may be considered. The culture already contains one, or the proper phage-precursor has not been used.

Isolates from plant tissue are always likely to contain phages. We may wonder if any bacterial cultures are actually phage-free. Recent investigations in this laboratory have revealed that phages may be removed from many secondary cultures by merely growing

the organism upon an agar-hardened medium. Surface colonies on poured plates commonly are free of phage contamination although the original culture did contain one.

As phages come into more general use among bacteriologists and are advanced beyond the stage of laboratory curiosities, we will become more fully informed of their merits and limitations as a means of distinguishing one species or strain of bacteria from another.

Summary

1. The need for a quick, reliable method of identifying species of bacteria by plant pathologists is indicated. The bacteriophage reaction offers great promise in this connection. Only phages which give complete lysis are suitable to use for identification purposes.

2. A method is outlined for the preparation and use of oat extract for the development of phages.

3. In tabular form, the reactions of a number of species of bacteria to specific and nonspecific phages are shown. The bacterial species selected include both plant and human pathogens.

4. Bacteriophages have been found to retain lytic activity when stored in a refrigerator at 8° C. for 6 years and, when preserved in sealed tubes at room temperature, for 12 years.

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